TOXICITY OF ELEMENTAL SULFUR FOR BRUCELLAE

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In previous reports we have described the toxicity of certain peptones and amino acids, particularly cystine, for brucellae (Schuhardt, Rode, Foster, and Oglesby, 1949; Schuhardt, Rode, and Oglesby, 1949). Subsequently, we found and reported that cystine *per se* is not toxic for these organisms, and proved that the observed toxicity of autoclaved cystine is due to a heat-induced breakdown product of cystine (Schuhardt, Rode, Oglesby, and Lankford, 1950). This report deals with the results of experiments designed to determine the nature of this toxic breakdown product of cystine.

EXPERIMENTAL RESULTS

The methods of testing for toxicity were the same as those previously described. Our culture of *Brucella abortus*, strain 1257, was the test organism in most of these experiments, and 0.5 per cent tryptose broth was the major test medium used.

In an effort to isolate the toxic factor, 6 g of L-cystine were dissolved in a minimum quantity of N HCl. This cystine solution was diluted to a volume of 6 liters with water, and the pH was adjusted to 2.3. The solution was autoclaved at 121 C for 30 minutes, allowed to cool, and adsorbed with 0.5 per cent norit. The norit was recovered by filtration, and the process of autoclaving and adsorbing with fresh norit was repeated on the original cystine solution a total of 6 times. The norit was combined, dried *in vacuo*, and eluted with three successive portions of pyridine. The combined pyridine eluate was concentrated under reduced pressure until the last traces of solvent were removed. This procedure yielded a dark brown syrupy residue containing benzene-soluble crystals. A benzene extract was reduced to dryness, and the crystals were purified by repeated crystallization from pyridine-water solution until a constant melting point of 118 to 119 C was obtained. These yellow, monoclinic crystals were shown to be sulfur.

Because of the extreme insolubility of sulfur in water, we were inclined to discount the probability of these crystals being the causative agent of the anti-brucella toxicity of autoclaved cystine. However, since sulfides were proved not to be the toxic factor, and since the toxicity could not be located in other fractions of the original pyridine eluate, experiments were undertaken to determine whether or not elemental sulfur was toxic for brucellae.

The sulfur was dissolved in acetone and dispersed in distilled water in concentrations ranging from 25 to 200 μg per ml. This procedure gave fairly stable, opalescent, colloidal dispersions of the sulfur which could be freed of solvent by

distillation and which remained stable when autoclaved. After varying periods of standing, a powdery white precipitate of elemental sulfur appeared. When a fresh preparation of this colloidally dispersed sulfur was added aseptically to McCullough and Dick (1943) medium, it proved toxic for *B. abortus* in concentrations as low as 0.06 μg of sulfur per ml. In a 0.1 per cent casamino acid medium, 0.14 μg per ml proved toxic; and in 0.5 per cent tryptose, 0.4 μg of the sulfur per ml of medium was toxic for this organism. In these and subsequent tests it became apparent that heat sterilization of the sulfur in the presence of the medium markedly increased the amount of sulfur required for antibrucella toxicity. This increase ranged from 45 to 360-fold depending upon the type of medium used.

In comparative studies of (1) colloidal sulfur toxicity, (2) autoclaved cystine toxicity, and (3) tryptose toxicity for brucellae, the three were found to possess practically identical properties. Each is bactericidal against strains of *B. abortus*. Each shows essentially the same antibrucella spectrum. The toxicity of each is influenced by the size of the inoculum of the test organism. The toxic factor in each instance can be adsorbed onto norit and eluted from this reagent with pyridine. Each toxicity is neutralized by blood serum, unwashed agar, a variety of reducing agents, and by filtered cystine. Heating at acid pH (1.5) has no effect on the 3 toxicities, but heating at alkaline pH (9.5) destroys each toxicity. Addition of sulfite, bisulfite, or hydrosulfite removes each of the toxicities as does treatment with H_2O_2. These results indicate the probability that elemental sulfur is the toxic breakdown product of autoclaved cystine, and probably also the antibrucella factor in toxic peptones.

Efforts have been made to identify the products of heat degradation of cystine to determine the mechanism whereby autoclaved cystine yields elemental sulfur. The presence of H_2S in freshly autoclaved cystine solutions is detectable by odor and confirmed by lead acetate. The H_2S gradually disappears upon standing, and, concurrently, a colloidal dispersion of elemental sulfur appears. Subsequently, a powdery precipitate begins to appear. In addition to H_2S, preliminary examination showed the presence of cysteine, pyruvic acid (isolated and identified as the 2,4-dinitrophenylhydrazone), and ammonia. These results are in accord with the observation of Routh (1938) that cysteine, H_2S, and S° are formed in boiled aqueous solutions of cystine. Routh, however, did not obtain evidence of deamination under these conditions.

Quantitative analyses were performed on many samples of autoclaved cystine. These gave fairly consistent data except for sulfur. Table 1 lists average yields of pyruvic acid, ammonia, and sulfur, which approximate anticipated equimolar concentrations. The pyruvic acid was determined by the Friedemann and Haugen (1943) modification of the method of Lu (1939). Ammonia was determined by the micromethod of Johnson (1941). However, since cyst(e)ine interferes with this determination, the method was applied to distillates obtained from the autoclaved cystine solution according to the method of Archibald (1943). Sulfur was determined by the method of Guthrie (1938). This determination gave yields of elemental sulfur ranging from 20 to 80 μg per mg of cystine autoclaved.

From the analytical data it appears that approximately 10 per cent of the cystine was degraded to S°, pyruvate, and ammonia. An additional undeter-

mined portion may have been converted to cysteine sulfinic acid. Thus, it may be estimated that between 10 and 20 per cent of the cystine was converted to products other than cysteine. This estimate was verified by assay in a hydrogen peroxide-treated casamino acids medium with *Brucella suis*, strain 32P, an organism which is resistant to sulfur toxicity and which utilizes cyst(e)ine but not

TABLE 1

*Occurrence of pyruvic acid, ammonia, and elemental sulfur in autoclaved cystine solution**

MATERIAL	MICROGRAMS DETECTED PER MG L-CYSTINE†		FRACTIONAL MOLAR RATIOS
	Autoclaved	Unautoclaved	
Pyruvic acid	71	0	0.097
Ammonia	14.7	0	0.104
Sulfur	40	0	0.150

* Solution of 1 mg L-cystine adjusted to pH 6.7 and autoclaved at 121 C for 30 min.
† Average yields of 4 to 8 experiments.

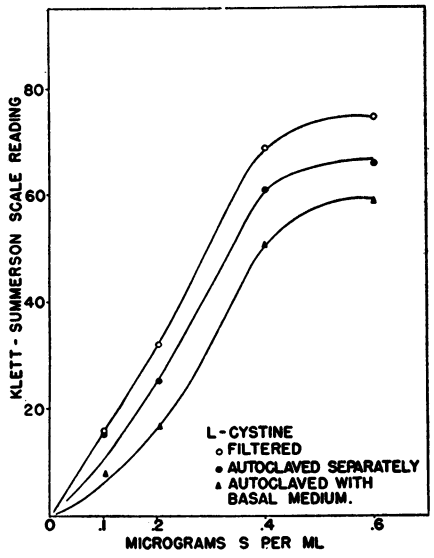

Figure 1. The effect of the method of sterilization on cystine utilization by *Brucella suis*. Growth turbidity was measured with the Klett-Summerson photoelectric colorimeter.
L-Cystine supplied as the only available sulfur source in a hydrogen peroxide-treated, casamino acid basal medium containing 0.2 per cent glucose.

cysteine sulfinic acid. Cultures were incubated at 37 C for 5 days after which they were killed by placing the tubes in flowing steam for 1 hour. Growth densities were determined by the Klett-Summerson photoelectric colorimeter, using the blue filter no. 42 (400 to 465 mμ). The decrease in growth response to cystine autoclaved separately, as compared with that to filtered cystine, indicates destruction of 13 to 25 per cent of the cystine, depending upon the portion of the curve (figure 1) used for comparison. The further decrease in growth response

when cystine was autoclaved in the glucose-containing basal medium suggests the participation of two different mechanisms of cystine destruction (Lankford, Swausch, and Ravel, 1947; Camien and Dunn, 1950).

As a consequence of reports in the literature and our own analytical studies, we believe the following scheme of cystine breakdown might be applicable:

1. A heat-induced rupture of the disulfide linkage with an associated dismutation (Toennies and Lavine, 1936) of a portion of the cystine to cysteine and cysteine sulfinic acid. According to Toennies and Lavine (1936) and Stahl *et al.* (1949), an intermediate unstable cysteine sulfenic acid is probable in this dismutation.

$$2\ R{-}S{-}S{-}R + 2\ H_2O + \text{Heat} \rightarrow 2\ R{-}SH + 2\ R{-}SOH$$
$$2\ R{-}SOH + \text{Heat} \rightarrow R{-}SO_2H + R{-}SH$$

2. The cysteine is degraded to pyruvic acid, ammonia, and hydrogen sulfide by way of an unstable imino acid (Smythe, 1942), or by way of *alpha*-amino acrylic acid (Nicolet, 1931).

$$R{-}SH + \text{Heat} \rightarrow H_2S + CH_2{=}\underset{\displaystyle NH_2}{\underset{|}{C}}{-}COOH \text{ or } CH_3{-}\underset{\displaystyle NH}{\underset{\|}{C}}{-}COOH$$

$$CH_3{-}\underset{\displaystyle NH}{\underset{\|}{C}}{-}COOH + H_2O \rightarrow CH_3{-}\underset{\displaystyle O}{\underset{\|}{C}}{-}COOH + NH_3$$

3. The hydrogen sulfide reacts with the excess cystine to form sulfur and additional cysteine (Andrews, 1928; Smythe, 1942).

$$H_2S + R{-}S{-}S{-}R \rightarrow 2\ R{-}SH + S^\circ$$

This last reaction is readily reversible, which fact might be expected to account for the ease of neutralization of sulfur toxicity by excess SH compounds. Other neutralization effects observed and reactions employed to correlate the toxicity of autoclaved cystine and toxic tryptose with that of elemental sulfur can also be explained on the basis of the reactivity of the sulfur molecule. For example, the neutralization of the antibrucella toxicity by reducing agents is presumably due to their functioning as hydrogen donors to convert toxic S° into nontoxic H_2S. The effectiveness of sulfite, bisulfite, and hydrosulfite in neutralizing antibrucella toxicity may be explained as a consequence of their reaction with S° to form thiosulfates and other oxidized sulfur products.

Further evidence of the reactivity of elemental sulfur is the previously mentioned observation that increased concentrations are required for antibrucella toxicity when the sulfur is autoclaved in the presence of the test medium. This reaction between colloidal sulfur and ingredients of the medium may not only detoxify the colloidal sulfur, but utilizable sulfur compounds may be produced. Figure 2 illustrates this situation wherein varying amounts of separately autoclaved colloidal sulfur were added aseptically to duplicate tubes of sterile, hydrogen peroxide-treated casamino acid basal medium containing no available source

of sulfur. One set of tubes was autoclaved after the addition of the sulfur. Each preparation was inoculated with 0.1 ml of a 48-hour tryptose broth culture of *Brucella suis*. The two curves in figure 2 indicate that, whereas colloidal sulfur in the concentration tested is not a utilizable sulfur source for this organism, it becomes so when autoclaved in the presence of the other ingredients of the basal medium.

It is more difficult to visualize the mechanism whereby cystine neutralizes the antibrucella toxicity of sulfur. This neutralization is competitive to a degree and might indicate that the elemental sulfur competes for enzyme systems concerned with the metabolism of this amino acid. However, it is possible that polysulfides (von Holmberg, 1908) might be formed when cystine reacts with elemental sul-

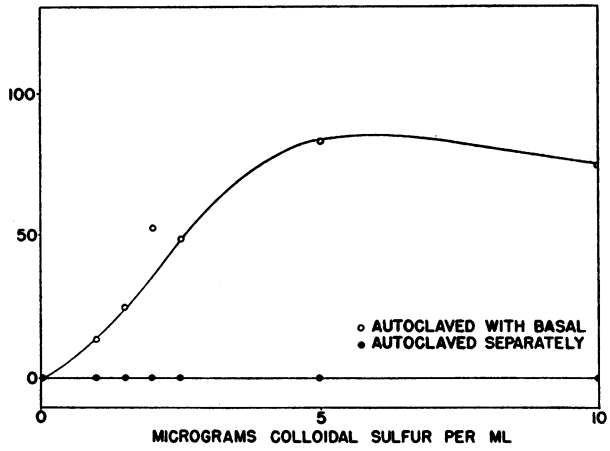

Figure 2. The effect of the conditions of sterlization on the utilization of elemental sulfur by *Brucella suis*.

Colloidal sulfur supplied as the only added source of sulfur in a hydrogen peroxide-treated casamino acid basal medium.

fur. It is tempting to speculate on the nature of the toxicity of elemental sulfur in terms of its reactivity with essential SH groups of certain enzyme systems or with reduced glutathione or other rH systems. However, further work is planned before conclusions about the mechanism of this sulfur toxicity are drawn.

SUMMARY AND CONCLUSIONS

Elemental sulfur has been isolated from autoclaved cystine solutions. Colloidal dispersions of this and other sulfur samples were toxic for certain strains of *Brucella* in concentrations as low as 0.06 μg per ml of culture medium.

This toxicity is comparable in all respects tested to the antibrucella toxicity of autoclaved cystine and toxic tryptose.

A possible mechanism for the heat degradation of solutions of cystine to elemental sulfur is described.

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